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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/132164> since 2016-01-28T16:33:39Z

Published version:

DOI:10.1016/j.cropro.2010.03.011

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Activity of benzothiadiazole on chrysanthemum yellows phytoplasma (*Candidatus Phytoplasma asteris*) infection in daisy plants

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Abstract

A commercial preparation of the plant resistance elicitor benzothiadiazole (BTH) (Bion, Syngenta Crop Protection) was tested for its capacity to induce systemic resistance against chrysanthemum yellow phytoplasma (CYP) infection in the *Chrysanthemum carinatum* plant. Following one 2.4 mM BTH application, plants were exposed to CYP-infective *Macrostelus quadripunctulatus* leafhoppers. Symptom development and phytoplasma multiplication in the test plants were measured over time. BTH application delayed symptom development and phytoplasma multiplication in treated plants compared with the control ones. CYP titre and symptom severity were significantly lower for the first two weeks post-inoculation in treated plants compared with the control ones, suggesting that systemic acquired resistance (SAR) induced by BTH in *C. carinatum* is temporary. Higher concentrations of BTH resulted in phytotoxic effects involving the whitening of apical leaves. BTH application was ineffective in reducing the transmission efficiency of CYP by its leafhopper vector. Actually, in both single and group transmission tests, the proportion of infected plants was similar among BTH-treated and untreated plants. The survival of *M. quadripunctulatus* was unaffected by feeding on BTH-treated daisy plants. Moreover, when leafhoppers were allowed to choose between treated and untreated plants, they showed no preference. We conclude that SAR induced in daisy plants by BTH has no detrimental effects on the vector leafhopper. If the activity of BTH against phytoplasmas is confirmed also on other

47 phytoplasma/host–plant associations, BTH applications might be included in new, more
48 environmentally friendly, integrated management strategies of phytoplasmoses.

49

50 **Keywords**

51 Benzothiadiazole, resistance elicitor, systemic acquired resistance, chrysanthemum yellows

52 phytoplasma, *Macrosteles quadripunctulatus*

53

54 **Introduction**

55 Phytoplasmas are wall-less pathogenic bacteria belonging to the class Mollicutes that are associated
56 with economically important diseases of fruit trees (pome fruit, stone fruit, coconut and grapes
57 among others), vegetables (lettuce, potato, tomato, etc.), ornamentals (herbaceous, trees and shrubs)
58 and weeds (Seemüller et al., 1998; Bertaccini et al., 2007). They are phloem-limited pathogens that
59 cause several different symptoms such as yellowing, stunting, proliferation, virescence, phyllody
60 and general decline. Phytoplasmas are transmitted in a persistent, propagative manner by
61 Hemipteran phloem-sucking insects in the Cicadellidae, Cixiidae, Derbidae, Delphacidae and
62 Psyllidae families (Weintraub and Beanland, 2006).

63 The control of phytoplasma diseases is difficult and relies mainly on insecticide treatments against
64 vector insects and the planting of healthy propagation material. Insecticides can be effective in
65 suppressing the vector population but only neonicotinoids actively prevent the transmission from
66 infectious insects visiting the crop (Saracco et al., 2008). Large scale use of insecticides (in
67 preventing the spread of phytoplasma diseases) has a negative impact on non-target arthropods,
68 especially mites (Waetermeulen et al., 1999) and, possibly, on pollinators (Vorwohl, 1977), and
69 represents a potential threat to human health. Planting phytoplasma-free stocks is advisable, but
70 control of vector populations is still required to prevent transmission during the vegetative season
71 (Morone et al., 2007). Traditional vector exclusion methods, such as the use of insect-proof nets,

though very effective if properly secured in place (Walsh et al., 2006), can be applied in protected crops but not open field conditions.

Systemic acquired resistance (SAR) is an inducible resistance mechanism in plants that provides resistance against plant pathogens. The treatment of plants with various agents (e.g., virulent or avirulent pathogens, nonpathogens, cell wall fragments, plant extracts and synthetic chemicals) can lead to the induction of resistance to subsequent pathogen attack. SAR by plant activators provided interesting results in the control of a broad spectrum of pathogens, such as bacteria, fungi and viruses (Oostendorp et al., 2001; Vallad and Goodman, 2004). In the vast majority of cases, SAR depends on the early increase of the endogenously synthesized signal salicylic acid and it is correlated with the immediate expression of a specific set of genes which includes those coding for pathogenesis-related-proteins (PR) (Pieterse and Van Loon, 2007). Several PR proteins (e.g. β -1,3 glucanases, chitinases, osmotin) possess anti-microbial activity and are thought to contribute to the state of resistance together with other PR (e.g. hydroxyproline rich glycoproteins, callose) that are important in cell wall structure. Among the synthetic inducers of plant disease resistance, acibenzolar-S-methyl, a benzothiadiazole (BTH), is a well-studied compound (Schurter et al., 1987; Friedrich et al., 1996) that has extensively been shown to induce SAR in many crops, such as monocots, solanaceous, leguminous and fruit trees (Gorlach et al., 1996; Buonauro et al., 2002; Dann and Deverall, 1995; Ishii et al., 1999).

Bressan and Purcell (2005) reported a significant effect of BTH in reducing X-disease phytoplasma transmission to *Arabidopsis thaliana* (L.) Heynh. by the leafhopper *Colladonus montanus* (Van Duzee), whereas Romanazzi et al. (2009) noticed a higher recovery rate from bois noir phytoplasma in grapevines treated with BTH and other resistance elicitors. Conflicting information is available on the effect of BTH on phytophagous insects. In most cases, the salicylic acid pathway involved in SAR development has only negligible effects on phytophagous insects (Inbar et al., 2001; Bi et al., 1997), but for some phloem feeders reduced fecundity and increased mortality have been reported following feeding on BTH-treated plants (Bressan and Purcell, 2005; Boughton et al., 2006).

98 The aim of this study was to investigate the effect of benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-
99 methyl ester (BTH), on the transmission of chrysanthemum yellows phytoplasma (CYP) to daisy
100 plants under controlled conditions. The association of *Chrysanthemum carinatum* (Schousboe)–
101 CYP–*Macrosteles quadripunctulatus* (Kirschbaum) represents an optimal model system because of
102 i) the short incubation of the phytoplasma in the plant and short latency in the vector, ii) rapid and
103 obvious symptom development, iii) extremely high transmission efficiency, iv) the polyvoltine
104 vector that can be easily reared throughout the year and v) the availability of detection and
105 quantification tools for the phytoplasma (Marzachi and Bosco, 2005). CYP is a strain of the
106 ‘*Candidatus* Phytoplasma asteris’ phytoplasma (16Sr-IB), which infects a variety of dicotyledonous
107 plants and is transmitted with different efficiencies by several species of leafhoppers (Bosco et al.,
108 2007). The activity of BTH on CYP disease was measured through the analysis of disease severity
109 and the quantification of CYP cells in infected, treated and untreated plants, by quantitative real
110 time PCR analysis (qRT-PCR).

111

112 **Materials and methods**

113 *Plants and BTH treatments*

114 *C. carinatum* plants were grown from seed, individually transplanted in 8 × 8 cm pots and
115 maintained in a greenhouse (20–25°C, photoperiod L16:D8). One month after sowing (when they
116 were 5–6 cm high), plants were sprayed with BTH solutions (Bion WG50, 50% active ingredient,
117 a.i., Syngenta Crop Protection) diluted with sterile water and applied as a foliar treatment to a run-
118 off point. Control plants were sprayed with water. After the treatment, plants were maintained in a
119 greenhouse under the conditions described above. Three different concentrations of BTH were
120 evaluated in preliminary assays, 1.2, 2.4 and 4.8 mM, to determine the concentration providing the
121 best results. The preliminary experiment was carried out twice. Three further experiments were then
122 performed using the 2.4 mM concentration only. In each experiment, 10 plants per treatment were
123 included: BTH-treated plants not exposed to CYP infection (BTH), untreated CYP-inoculated

124 plants (CYP), control plants untreated and unexposed to CYP (C) and BTH-treated and CYP-
125 infected plants (BTH-CYP).

126

127 *Phytoplasma and vector insect*

128 The ‘*Candidatus* Phytoplasma asteris’ (strain 16Sr-IB), originally isolated from *Argyranthemum*
129 *frutescens* (L.) Schultz-Bip plants in Liguria (Italy) (Conti and Mela, 1987), was maintained on the
130 daisy (*C. carinatum*) by vector transmission.

131 Healthy colonies of *M. quadripunctulatus* were reared on potted oat plants inside Plexiglas and
132 nylon cages in growth chambers at 25°C (photoperiod L16:D8) and checked by nested PCR using
133 the R16F2/R2 followed by R16F1/R1 (I) primers (Lee et al., 1993, 1994) to verify phytoplasma
134 absence.

135 For transmission experiments, the third- to fifth-instar nymphs were fed for one week of the
136 acquisition access period (AAP) on CYP-infected plants, transferred onto healthy oat plants for two
137 weeks to complete the latency period (LP) and then transferred individually to 10 daisy plants,
138 confined inside glass cylinders, for each elicitor and control treatment, for an inoculation access
139 period (IAP) of three days. Males and females of *M. quadripunctulatus* were used in the
140 transmission experiments since both genders proved to transmit CYP with 100% efficiency under
141 the experimental conditions described above (Bosco et al., 2007). Test plants were exposed to
142 vectors one week after BTH treatment (about five weeks after sowing). Insects were then removed
143 and plants were drench-treated with the systemic insecticide thiamethoxam (Actara, Syngenta Crop
144 Protection), 7 mg a.i. per plant, to kill nymphs hatched from eggs eventually laid by vector females.
145 Test plants were maintained in the greenhouse for about one month. Daisy plants treated with water
146 and exposed to vectors were used as transmission controls. Treated plants not exposed to vectors
147 were used as treatment controls. Apical leaves of five daisy test plants were sampled at six, 11, 13
148 and 20 days after the end of the inoculation (dpi) from the same test plants. Total DNA was

149 extracted and CYP DNA quantified by qRT-PCR. The quantification of phytoplasma cells was not
150 performed in the two preliminary experiments where only symptom severity was evaluated.

151

152 *Choice test*

153 In a choice experiment, eight healthy daisy plants (5–6 cm high) were sprayed to the run-off point
154 with 2.4 mM BTH and eight healthy daisy plants were sprayed with water as a control. After one
155 week, treated and untreated plants were set interspersed inside a Plexiglas and nylon cage (80 × 80
156 × 60 cm). Following an AAP of seven days on untreated source plants and an LP on oat as
157 described above, 10 infective adult vectors were introduced into the cage for an IAP of three days;
158 vectors were free to fly and feed on all the plants. The cage was maintained in a climatic chamber at
159 25°C (photoperiod L16:D8). At the end of the IAP, insects were counted and removed. Test plants
160 were treated with insecticide and transferred to the greenhouse for about one month or until the
161 appearance of symptoms. Infection of the plants was established on the basis of symptoms. The
162 experiment was performed twice.

163

164 *No-choice test*

165 Sixteen daisy plants (5–6 cm high) were treated with 2.4 mM BTH to the run-off point and 16
166 plants were treated with water as a control. After one week, BTH-treated and control plants were
167 separately placed inside two Plexiglas and nylon cages (80 × 80 × 60 cm). Following an AAP on
168 CYP source plants of seven days and an LP on oat as described, 10 infective adult vectors were
169 introduced into each cage for an IAP of three days on the test plants; vectors were free to fly and
170 feed on all plants. The cages were maintained in a climatic chamber at 25°C (photoperiod L16:D8).
171 At the end of the IAP, insects were counted and removed. Plants were treated with insecticides and
172 transferred to the greenhouse for about one month. Infection of the plants was established on the
173 basis of symptoms. The experiment was performed twice.

174

175 *Symptom evaluation*

176 The severity of the symptoms of test plants was evaluated three times a week between 11 and 32
177 dpi, and plants were classified into five classes of severity: 0 = no symptoms, 1 = yellowing of the
178 apex, 2 = yellowing and distortion of the apex, 3 = apex growth stunt, 4 = severe yellowing and
179 dwarfing of the whole plant and 5 = plant death.

180

181 *Phytoplasma concentration: DNA extraction and qRT-PCR*

182 The activity of BTH on CYP was quantified by assessing phytoplasma titre in five plants from each
183 treatment. Total DNA was extracted from CYP-infected daisy leaves (0.1 g) using the PureLink
184 Plant Total DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's
185 protocol and eluted in 50 µL of kit elution buffer. Total DNA was extracted from healthy daisy
186 plants using the same protocol. The concentration of DNA extracts from CYP-infected daisies and
187 healthy controls was measured with a NanoDrop 1000 Spectrophotometer (Thermo Fisher
188 Scientific, Wilmington, DE, USA). Diluted samples (1 ng/µL in sterile double-distilled water) were
189 analysed in triplicate in qRT-PCR assays. CYP DNA in each sample was measured as the number
190 of CYP cells per ng of plant DNA (Marzachì and Bosco, 2005).

191 The absolute quantification of CYP was achieved by comparing the CTs of the samples with those
192 of three dilutions of a plasmid, pOP74, containing the phytoplasma 16S rDNA target sequence. One
193 femtogram of pOP74 contained 194 molecules of plasmid, with each containing a single copy of the
194 CYP 16S rDNA gene. Because this gene is present in two copies in phytoplasma genomes, one
195 femtogram of pOP74 corresponded to 97 CYP cells (Marzachì and Bosco, 2005). The absolute
196 quantification of daisy plant DNA was achieved by comparing the CTs of the samples with those of
197 four dilutions of healthy daisy DNA. All samples were run on triplicate.

198

199 *Data analysis*

200 Symptom evaluation. At each rating date, the means and standard errors of severity class were
201 calculated for treated and control plants and pairwise t tests were performed to compare symptom
202 severity between treatments, according to Alexander et al. (1993). Results from the transmission
203 experiments (positive/negative plants) were analysed using the χ^2 test.

204 Phytoplasma concentration. For the analyses, the concentration of CYP in each plant was expressed
205 as the difference between the logarithm concentration of CYP and that of daisy plant DNAs.
206 Finally, CYP concentration was expressed as cell number per ng of plant DNA. After comparing
207 the phytoplasma titre measured at each sampling date in the three quantification experiments, data
208 were pooled since they did not differ significantly among experiments. To compare phytoplasma
209 titre measured at different dpi in BTH-treated and control plants, a two-way ANOVA for date and
210 treatment was performed. The ANOVA, t test and χ^2 test were performed using Jandel SigmaPlot
211 11.0 (Systat Software, Inc, San Jose, CA, USA).

212

213 **Results**

214 *Transmission results and symptom severity*

215 Following inoculation with CYP-infective leafhoppers, 16 out of 20 plants treated with 1.2 mM
216 BTH, 14 out of 20 plants treated with 2.4 mM BTH and 19 out of 20 plants treated with 4.8 mM
217 BTH became infected. Among the untreated control plants, 16 out of 20 became infected. The
218 proportion of infected/uninfected plants between BTH-treated and untreated plants did not differ
219 significantly. Moreover, the 4.8 mM BTH treatment caused phytotoxic effects on some plants,
220 which exhibited an unusual whitening of the apical leaves and inhibited plant growth compared
221 with the controls. These effects lasted from 14 dpi until the end of the experiment. Symptom
222 severity recorded from 11 to 32 dpi on plants treated with different BTH concentrations and
223 untreated infected control plants is shown in Fig. 1. Consequently, in the following experiments the
224 2.4 mM BTH treatment was chosen. Twenty-five out of 30 2.4 mM BTH-treated plants and 29 out
225 of 30 untreated plants became infected in the remaining experiments. Overall, following inoculation

226 with CYP-infective leafhoppers, 39 out of 50 plants treated with 2.4 mM BTH and 45 out of 50
227 untreated plants became infected, but the difference was not significant ($\chi^2 = 1.860$; $P = 0.173$). The
228 results of the five experiment repeats with 2.4 mM BTH are summarised in Fig. 2. Symptom
229 severity recorded from 11 to 32 dpi on 50 plants treated with 2.4 mM BTH and the 50 untreated
230 infected control plants is shown in Fig. 3. Overall, test plants treated with 2.4 mM BTH showed
231 lower symptom severity, but this difference was significant only at 11, 14 and 17 dpi ($P = 0.013$, P
232 $= 0.001$ and $P = 0.005$, respectively) (Fig. 3).

233 In one experiment, two 2.4 mM BTH-treated plants showed clear symptoms until 31 dpi (class 3
234 symptoms), but afterwards the symptoms completely disappeared. The recovery of these plants was
235 checked by nested PCR using the R16F2/R2 followed by R16F1/R1 (I) primers (Lee et al., 1993,
236 1994) and they proved to be phytoplasma-negative.

237

238 *Choice test*

239 When infective leafhoppers were allowed to feed freely on 2.4 mM BTH-treated and control plants,
240 14 out of 32 BTH-treated plants and 12 out of 32 control plants became infected; however, the
241 difference was not significant. Eight and nine live insects were counted at the end of the two repeats
242 of the experiment.

243

244 *No-choice test*

245 When infective leafhoppers were allowed to feed separately on 16 BTH-treated or control plants, 21
246 out of 32 control plants and 23 out of 32 BTH-treated plants became infected; however, the
247 difference was not significant. Eight and nine live insects were counted at the end of the two repeats
248 of the experiment for the treated plants, and seven and nine live insects were counted at the end of
249 the two repeats of the experiment for the untreated plants.

250

251 *CYP titre*

Phytoplasma titre in plants treated with 2.4 mM BTH was significantly lower than that of control plants ($P<0.001$). Phytoplasma titre significantly increased over time from 6 to 20 dpi ($P<0.001$) in treated and control plants. There was no significant interaction between date and treatment. Mean numbers of CYP cells per ng of plant DNA in treated and control plants are shown in Table 1.

Discussion

BTH has been largely employed to activate SAR for the control of plant disease caused by several pathogenic fungi, bacteria and viruses. However, little is known about its activity towards phytoplasma diseases and, to our knowledge, only two reports are available on the activity of this chemical on phytoplasmas: one towards X-disease phytoplasma transmission to *A. thaliana* studied under controlled conditions (Bressan and Purcell, 2005), and the other on grapevine bois noir phytoplasma (Stolbur phytoplasma or '*Candidatus* Phytoplasma solani') under field conditions (Romanazzi et al., 2009). Our study demonstrated for the first time that phytoplasma multiplication and symptom development are partially hampered in the host plant by BTH treatment. CYP symptom development and multiplication were both delayed 13–17 dpi under our experimental conditions. Later, BTH elicitation against phytoplasma infection seemed to be lost. This suggests that BTH elicitation against CYP is temporary and tends to disappear over time. In our experiments, we treated test plants only once, one week before insect transmission, and we cannot exclude that a further application might have delayed phytoplasma multiplication and symptom severity later in the infection process. Actually, Buonauro et al. (2002) found that, under field conditions, the highest efficacy of this elicitor against *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye was obtained by spraying pepper plants 6–7 times every 8–12 days. YuHong et al. (2006) reported that the induced resistance against downy mildew in cucumber plants persisted for 15 days. It is known that BTH-elicited resistance lasts longer in monocots than dicots (Oostendorp et al., 2001; Vallad and Goodman, 2004), and that the temporary activity of BTH we recorded in CYP-infected daisy

plants is consistent with this. Interestingly, BTH application was effective in delaying CYP multiplication and symptom development but not in reducing transmission efficiency of CYP by its leafhopper vector. Actually, in both single and group transmission tests, the proportion of infected plants was similar among BTH-treated and untreated plants. This result is consistent with the fact that the survival of *M. quadripunctulatus* was unaffected by the feeding period on daisy BTH-treated plants. On the contrary, the reduced survival of *C. montanus* on *A. thaliana* BTH-treated plants suggests that SAR in this plant might have some detrimental effect on this leafhopper, inhibiting vector feeding and thereby phytoplasma transmission efficiency (Bressan and Purcell, 2005). Considering that the different pathways might not be similarly regulated in all systems (Maleck and Dietrich, 1999), it seems that the systemic response induced by Bion on insect herbivores could vary among plant and insect species. Our results are, therefore, consistent with other studies reporting that SAR had a slight or negligible effect on phytophagous insects such as some cotton pests or the silverleaf whitefly (Inbar et al., 2001; Bi et al., 1997). Other studies noticed some resistance against the silverleaf whitefly induced by BTH. The resistance consists in nymph number reduction and in a delay in insect development (Nombela et al., 2005; Muniz et al., 2009).

We recorded lower phytoplasma multiplication in BTH-elicited plants. Similarly, SAR induced by BTH or plant growth-promoting rhizobacteria was found to suppress the replication of cucumber mosaic virus (Anfoka, 2000, Raupach et al., 1995). Moreover, our study also showed that BTH-treated plants had less severe symptoms. Therefore, we suggest that the lower phytoplasma titre in the elicited plants is directly correlated to the delay in symptom expression. These results suggest that BTH could induce resistance with an indirect or direct mechanism. An indirect activity, through the activation of SAR, is more likely to occur because BTH is known to induce the expression of SAR genes (Friedrich et al., 1996; Gorlach et al., 1996; Lawton et al., 1996). Until now, Bion is known to activate a plant's defence system only and not directly on the pathogen. A direct effect on phytoplasma multiplication cannot be excluded *a priori*. Because the chemical persistence of BTH

303 does not exceed five days (Buonaurio et al., 2002), whereas we recorded phytoplasma
304 multiplication and symptom expression for up to two weeks, an indirect mechanism of action is
305 suggested.

306 We observed that, under our conditions, BTH displayed some phytotoxic effects when applied at a
307 4.8 mM concentration. This is unsurprising given that several authors reported phytotoxic effects at
308 different concentrations in different crop species/cultivars (Iriti and Faoro, 2003; Amzalek and
309 Cohen, 2007; Perez et al., 2003; Bigirimana and Höfte, 2002).

310 Two daisy plants treated with 2.4 mM BTH and infected by CYP following insect transmission
311 completely recovered from the disease after one month. This finding, although not repetitive, is
312 peculiar because in our experience we have never observed such a recovery. This complete
313 recovery involved the disappearance of CY phytoplasmas, as demonstrated by nested PCR assays,
314 and was likely to be because of the BTH application. A similar recovery-inducing effect has been
315 reported for BTH-treated grapevines (Romanazzi et al., 2009).

316 The practical application of resistance elicitors towards phytoplasma diseases is in its infancy but,
317 given the difficulties in controlling phytoplasma diseases (relying mainly on insecticide treatments
318 against the vectors and roguing infected plants), the use of BTH is a promising new tool that could
319 be implemented in an integrated pest management strategy. Main limitations are represented by the
320 need of repeated applications and by the only partial tolerance that may be of low agronomical
321 interest, depending on the crop. In the light of avoiding repeated applications some biotic elicitors,
322 mycorrhizal fungi and rhizobacteria, seem to be promising (D'Amelio et al., 2007). Our results,
323 obtained on an experimental model pathosystem, should now be tentatively transferred to more
324 important crop systems (e.g., vineyards and orchards) before designing new integrated management
325 strategies that will eventually allow the reduction of insecticide applications for a more
326 environmentally friendly control of phytoplasmoses.

327

328 **Acknowledgements**

329 This work has been supported by the Piedmont Region within the project "Valutazione dell'azione
330 di microrganismi rizosferici ed elicitori di resistenza sull'infezione da fitoplasmi in un sistema
331 modello (CIPE 2006)".

332

333 **References**

- 334 Alexander, D., Goodman, R.M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox,
335 D., Ahl-Goy, P., Luntz, T., Wad, E., Ryals, J., 1993. Increased tolerance to two oomycete
336 pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad.
337 Sci. USA 90, 7327–7331.
- 338 Amzalek, E., Cohen, Y., 2007. Comparative efficacy of systemic acquired resistance-inducing
339 compounds against rust infection in sunflower plants. Phytopathology 97, 179–186.
- 340 Anfoka, G.H., 2000. Benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester induces systemic
341 resistance in tomato (*Lycopersicon esculentum* Mill. cv. Volledung) to cucumber mosaic
342 virus. Crop Protection 19, 401–405.
- 343 Bertaccini, A., 2007. Phytoplasmas: diversity, taxonomy and epidemiology. Frontiers in Bioscience
344 12, 673–689.
- 345 Bi, J.L., Murphy, J.B., Felton, G.W., 1997. Does salicylic acid act as a signal in cotton for induced
346 resistance to *Helicoverpa zea*?. J. Chem. Ecol. 23, 1805–1818.
- 347 Bigirimana, J., Höfte, M., 2002. Induction of systemic resistance to *Colletotrichum lindemuthianum*
348 in bean by a benzothiadiazole derivative and rhizobacteria. Phytoparasitica 30, 159–168.
- 349 Bosco, D., Galetto, L., Leoncini, P., Saracco, P., Raccach, B., Marzachi, C., 2007. Interrelationships
350 Between ‘*Candidatus* Phytoplasma asteris’ and Its Leafhopper Vectors (Homoptera:
351 Cicadellidae). J. Econ. Entomol. 100(5), 1504-1511.
- 352 Boughton, A.J., Hoover, K., Felton, G.W., 2006. Impact of chemical elicitor applications on
353 greenhouse tomato plants and population growth of the green peach aphid, *Myzus persicae*.
354 Entomol. Exp. Appl. 120, 175–188.

355 Bressan A., Purcell A.H., 2005. Effect of benzothiadiazole on transmission of X-disease
 356 phytoplasma by the vector *Colladonus montanus* to *Arabidopsis thaliana*, a new experimental
 357 host plant. Plant Dis. 89, 1121–1124.

358 Buonauro, R., Scarponi, L., Ferrara, M., Sidoti, P., Bertona, P., 2002. Induction of systemic
 359 acquired resistance in pepper plants by acibenzolar-S-methyl against bacterial spot disease.
 360 Eur. J. Plant Pathol. 108, 41–49.

361 Conti, M., Mela, L., 1987. Il giallume della margherita (*Chrysanthemum frutescens*), micoplasmosi
 362 tipica della riviera ligure. La Difesa delle Piante 10, 171–178.

363 D'Amelio R., Massa N., Gamalero E., D'Agostino G., Sampo S., Berta G., Faoro F., Iriti M., Bosco
 364 D., Marzachi C., 2007. Preliminary results on the evaluation of the effects of elicitors of plant
 365 resistance on chrysanthemum yellows phytoplasma infection. Bull. Insectol. 60, 317–318.

366 Dann, E.K., Deverall, B.J., 1995. Effectiveness of systemic resistance in bean against foliar and
 367 soil-borne pathogens as induced by biological and chemical means. Plant Pathol. 44, 458–
 368 466.

369 Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M.G., Meier, B., Dincher, S.,
 370 Staub, T., Uknes, S., Metraux, J.P., Kessmann, H., Ryals, J., 1996. A benzothiadiazole
 371 derivative induces systemic acquired resistance in tobacco. Plant J. 10, 61–70.

372 Görlach, J., Volrath, S., Knaufbeiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M.,
 373 Staub, T., Ward, E., Kessmann, H., Ryals, J., 1996. Benzothiadiazole, a novel class of
 374 inducers of systemic acquired resistance, activates gene expression and disease resistance in
 375 wheat. Plant Cell 8, 629–643.

376 Inbar, M., Doostdar, H., Gerling, D., Mayer, R.T., 2001. Induction of systemic acquired resistance
 377 in cotton by BTH has a negligible effect on phytophagous insects. Entomol.. Exp.. Appl. 99,
 378 65–70.

379 Iriti, M., Faoro, F., 2003. Benzothiadiazole (BTH) induces cell-death independent resistance in
 380 *Phaseolus vulgaris* against *Uromyces appendiculatus*. J. Phytopathol. 151, 171–180.

381 Ishii, H., Tomita Y., Horio T., Narusaka Y., Nakazawa Y., Nishiumura K., Iwamoto S., 1999.
 382 Induced resistance of acibenzolar-S-methyl (CGA 245704) to cucumber and Japanese pear
 383 diseases. *Eur. J. Plant Pathol.* 105, 77–85.

384 Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., Ryals
 385 J., 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the
 386 systemic acquired resistance signal transduction pathway. *Plant J.* 10, 71–82.

387 Lee, I.M., Hammond, R.W., Davis, R.E., Gundersen, D.E., 1993. Universal amplification and
 388 analysis of pathogen 16SrDNA for classification and identification of mycoplasma-like
 389 organisms. *Phytopathology* 83, 834–842.

390 Lee, I.M., Gundersen, D.E., Hammond, R.W., Davis, R.E., 1994. Use of mycoplasma-like organism
 391 (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed MLO
 392 infection in a single host plant. *Phytopathology* 84, 559–566.

393 Maleck, K., Dietrich, R.A., 1999. Defense on multiple fronts: how do plants cope with diverse
 394 enemies? *Trends in Plant Sci.* 4, 215–219.

395 Marzachi, C., Bosco D., 2005. Relative quantification of chrysanthemum yellows (16Sr I)
 396 phytoplasma in its plant and insect host using real-time PCR. *Mol. Biotechnol.* 30, 117–127.

397 Morone, C., Boveri, M., Giosue, S., Gotta, P., Rossi, V., Scapin, I., Marzachi, C., 2007.
 398 Epidemiology of Flavescence doree in vineyards in northwestern Italy. *Phytopathology* 97,
 399 1422–1427.

400 Muñiz, M.; Nombela, G.; Saygili, H.; Sahin, F.; Aysan, Y., 2009. Research on tomato resistance to
 401 the virus-transmitter whitefly *Bemisia tabaci* undertaken during the last years in Madrid
 402 (Spain). *Acta Hortic.*, 808, 175-179.

403 Nombela, G.; Pascual, S.; Aviles, M.; Guillard, E.; Muñiz, M., 2005. Benzothiadiazole induces
 404 local resistance to *Bemisia tabaci* (Hemiptera: Aleyrodidae) in tomato plants. *J. Econ.*
 405 *Entomol.* 98 (6), 2266-2271.

406 Oostendorp, M., Kunz, W., Dietrich, B., Staub, T., 2001. Induced disease resistance in plants by
 407 chemicals. *Eur. J. Plant Pathol.* 107, 19–28.

408 Perez, L., Rodriguez, M. E., Rodriguez, F., Roson, C., 2003. Efficacy of acibenzolar-S-methyl, an
 409 inducer of systemic acquired resistance against tobacco blue mould caused by *Peronospora*
 410 *hyoscyami* f. sp. *tabacina*. *Crop Protection* 22, 405–413.

411 Pieterse C.M.J., Van Loon L.C., 2007. Signalling cascades involved in induced resistance. In:
 412 Walters, D., Newton, A., Lyon, G. (Eds.), *Induced resistance for plant disease control: a*
 413 *sustainable approach to crop protection*. Blackwell Publishing, Oxford, UK, pp.65-88.

414 Raupach, G.S., Murphy, J.F., Kloepper, J.W., 1995. Biological control of cucumber mosaic
 415 cucumovirus in *Cucumis sativus* L., by PGPR-mediated-induced systemic resistance.
 416 *Phytopathology* 85, 1167.

417 Romanazzi G., D’Ascenzo, D., Murolo, S., 2009. Field treatment with resistance inducers for the
 418 control of grapevine bois noir. *J. Plant Pathol.* 91, 677-682.

419 Saracco P., Marzachì C., Bosco D., 2008. Activity of some insecticides in preventing transmission
 420 of chrysanthemum yellows phytoplasma (*Candidatus* *Phytoplasma asteris*) by the
 421 leafhopper *Macrostelus quadripunctulatus* Kirschbaum. *Crop Protection* 27, 130–136.

422 Schurter, R., Kunz, W., Nyfeler, R., 1987. Process and a composition for immunizing plants against
 423 diseases. US Patent 4931581.

424 Seemüller, E., Marcone, C., Lauer, U., Ragozzino, A., Göschl, M., 1998. Current status of
 425 molecular classification of the phytoplasmas. *J. Plant Pathol.* 80, 3–26.

426 Vallad, E.G., Goodman, R.M., 2004. Systemic acquired resistance and induced systemic resistance
 427 in conventional agriculture. *Crop Science* 44, 1920–1934.

428 Vorwohl, G., 1977. The importance of the grapevine as a pollen plant for the honeybee. *Apidologie*,
 429 8 (3), 237-257.

430 Walsh, K.B., Guthrie, J.N., White, D. T., 2006. Control of phytoplasma diseases of papaya in
 431 Australia using netting. *Australasian Plant Pathol.* 25, 49-54.

- 432 Waetermeulen, X., van Girard, D., Legras, S., 1999. Influence on mite fauna of applications
433 targeted against the flavescence doree vector, *Scaphoideus titanus* (Ball). in vineyards. In:
434 Proceedings of the Fifth International Conference on Pests in Agriculture, Part 2, Montpellier,
435 France, 7–9 December 1999, pp. 409-416.
- 436 Weintraub, P.G., Beanland, L., 2006. Insect vectors of phytoplasmas. Ann. Rev. Entomol. 51, 91–
437 111.
- 438 YuHong, L., Peng, C., ZhiHui, C., HuiFang, D., 2006. Induction of resistance to downy mildew by
439 oxalic acid or BTH in cucumber seedlings and the accumulation of pathogenesis-related
440 proteins in intercellular fluids. Acta Phytopathol. Sinica 36, 238–243.
- 441

442 **Table 1.** CYP concentration in BTH-treated and control daisy plants expressed as the mean \pm
 443 standard error (SE) of cells per ng of plant DNA. Plants were sampled at six, 11, 13 and 20 dpi. n =
 444 sample size. Within rows, values followed by the same letter do not differ significantly ($P < 0.05$).
 445

	CYP	BTH + CYP
dpi	mean cells/ ng plant DNA \pm SE (n)	mean cells/ ng plant DNA \pm SE (n)
6	$5.17 \times 10^3 \pm 1.05 \times 10^3$ (7) a	$2.43 \times 10^3 \pm 3.17 \times 10^2$ (7) b
11	$1.56 \times 10^5 \pm 2.5 \times 10^4$ (12) a	$8.45 \times 10^4 \pm 1.93 \times 10^4$ (10) b
13	$1.38 \times 10^5 \pm 2.53 \times 10^4$ (12) a	$6.88 \times 10^4 \pm 1.61 \times 10^4$ (11) b
20	$2.96 \times 10^5 \pm 4.29 \times 10^4$ (11) a	$2.02 \times 10^5 \pm 1.51 \times 10^4$ (9) a

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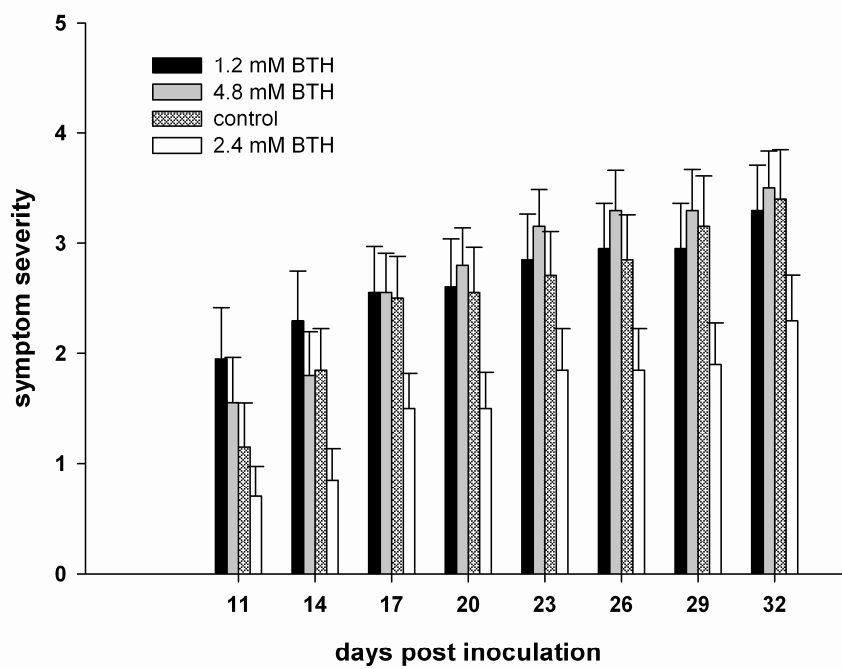
451 **Figures captions**

452 **Figure 1.** CYP symptom severity observed in the preliminary experiments in BTH-treated and
453 control plants. Symptoms were observed from 11 until 32 dpi on 20 plants at each observation date.

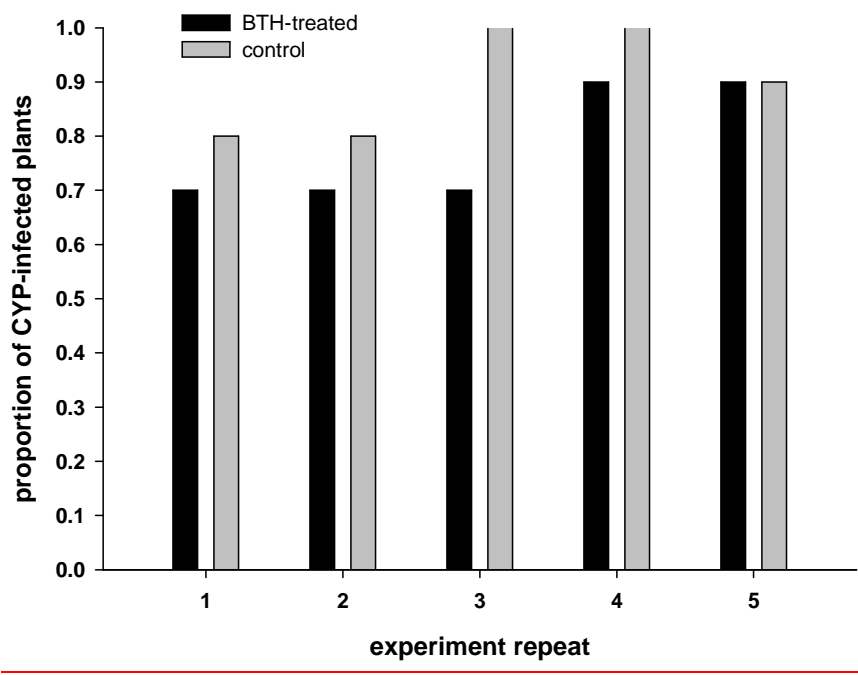
454 **Figure 2.** Proportion of CYP-infected daisy plants observed in the five experiment repeats on 2.4
455 mM BTH-treated and control plants.

456 **Figure 3.** CYP symptom severity recorded on 2.4 mM BTH-treated and control plants observed
457 from 11 until 32 dpi. Asterisks indicate significant differences between the two plant treatments
458 according to the *t* test. Fifty plants were observed at each dpi.

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